Aus dem Institut für Hygiene der Universität Düsseldorf
und dem Medizinischen Institut für Umwelthygiene
an der Universität Düsseldorf

Direktor: Professor Dr. med. Hans-Werner-Schlipköter

# Urinary Excretion of Biogenic Amines and Metabolites in Rats: Changes After Chronic Exposure to Controllable and Uncontrollable Electrical Footshock.

Dissertation

zur Erlangung des Grades eines Doktors der Medizin
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# Urinary Excretion of Biogenic Amines and Metabolites in Rats: Changes After Chronic Exposure to Controllable and Uncontrollable Electrical Footshock.

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*Throughout this article following abbreviations are used
NA=Noradrenaline (Norepinephrine), A=Adrenaline (Epinephrine)
MNA=Normetanephrine, DHPG=3-4-Dihydroxyphenylglycol,
HMPG=4-Hydroxy-3-metoxyphenylglycol, p-Tyra=p-Tyramine,
m-Tyra=m-Tyramine, GABA=Gammaaminobutryc acid, DA=Dopamine
HMPE=4-Hydroxy-3-metoxyphenylethanol, HVA=Homovanillin-
mandelic acid, DHPAA=3-4-Dihydroxyphenylacetic acid,
VMA=4-Hydroxy-3-methoxymandelic acid (Vanillylmandelic acid)
5-HT=5-Hydroxytryptamine, 5-HIAA=5-Hydroxyindolacetic acid,
TMS=Trimethylsilyl, HFB=Heptafluorobutyryl, AN=Acetonitrile,
MSTFA=N-methyl-N-trimethylsilyltrifluoroacetamid,
MBHFBA=N-methyl-bis-heptafluorobutyramid, GC-MS(F)=Gas-
chromatography mass spectrometry (massfragmentography)

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Abstract

A yoked control design with Wistar-rats was used to answer the following questions:

1. Is there an observable effect on urinary biogenic amines in a chronic stress experiment, depending on whether the animals can or cannot control the stressor?
2. Is there a common change over the time on the amount of urinary biogenic amines and metabolites output?
3. Is there a differential metabolic excretion profile for these compounds, indicating differential biochemical time processes?

Two groups of animals were exposed daily to a shuttle-box situation over a period of thirty days. One group - the manager animals - was allowed to escape from shock, i.e. to control the aversive stimuli of the situation, whereas the other group - the yoked animals - was not.
The urinary output of the animals was sampled every day for the simultaneous determination of 7 unconjugated urinary biogenic amines and metabolites.

The pooled catecholamines did not show statistical significant differences between the two groups.

A time effect $p \leq 0.001$ based on a three stage (cubic) time model is shown most pronounciated by the catecholamines and metabolites.

On the other hand p-tyramine and GABA exhibit an oscillating and/or increasing excretion time pattern, indicating a somewhat higher excretion by the yoked animals.

Key words:

Rat urinary biogenic amines and metabolites, p-tyramine
Uncontrollable electric footshock
Gaschromatography-Massfragmentography

Kobelt, R., Bisping, R.:

Urinary excretion of biogenic amines and metabolites in rats: changes after exposure to controllable and uncontrollable electric footshock.

It is generally agreed in the literature, that the amount of controllability an organism is allowed to exert on aversive stimuli is an important factor in determining the intensitiy and quality of its individual stress reponse. (For a review see McFerran & Breen, 1979). Typically, an effective stressor provokes a broad spectrum of differential responses on the physiological and behavioural level.

Despite the fact, that there is no general agreement in the literature, regarding the exact definition of stress, we make our assumptions on the basis of the working hypothesis, that the stress reponse represents the ability of the organism to adapt itself to cope with harmful situations (Selye, 1975). It can be shown experimentally, that the experience of in-contingency between the organism adaptive responses and their impact on the stressful event (i.e. uncontrollability) has an interfering effect on performance in situations, where controllability is allowed.

Seligman (Seligman, 1975) has formulated a cognitive theory of 'learned helplessness' to interpret this interference phenomenon: expectancies and attributions the organism develops in an uncontrollable situation are generalized to other - controllable - situations, which at the worst result in a state of pathological behavioural dysfunction.

Weiss (Weiss et al., 1976) has argued, that the interference effect may be due to the conditioning of certain responses in the uncontrollable situation, which are not compatible with another effective control in other situations.

Further Weiss et al. (1970, 1980) proved, that uncontrollable electric shock in a yoked-control design with rats led to a depletion of brain noradrenaline (NA), whereas animals, able to control shock by escaping, did not show this effect. The 'helplessness effect' may thus be mediated by a specific neurochemical state of the brain rather than by cognitive functions. Such functions are yet not well understandable in terms of animal behaviour.

Another important factor for the stress response - in addition to controllability - is the time variable. It is possible to show, that neurochemical changes following acute stress are quite different to those correlated with chronic stress (Weiss et al., 1975); Kvethansky et al., 1976, 1977). For a better understanding of severe states of pathological dysfunction where chronic stress may be involved as a predisposing factor, e.g. cardiovascular deseases, depression deseases or even cancer (Sklar & Anisman, 1981; Anisman & Zacharko, 1982) it would appear the animal model of chronic stress is more appropriate than an acute stress model.

Of course, a non-invasive technique has to be chosen to show the influence of the time variable when more than just the final result of the treatment (i.e. urine analysis) is to be evaluated. The biochemical analysis of urine metabolites in particular those of the catecholamine-system seems to be a reasonable way to demonstrate chronic stress effects as the various studies with animals and humans indicate (Leduc, 1961; Le Blanc, 1976; Benes, 1978; Greenspan et al., 1969).

On the basis of these considerations we developed an experimental design to answer the following questions:

1. Is there an observable effect on urinary biogenic amines in a chronic stress experiment with rats, depending on whether the animals can or cannot control the stressor?
2. Is there a common change over the time on the amount of urinary biogenic amines and metabolites output?
3. Is there a differential metabolic excretion profile for these compounds, indicating differential biochemical time processes?

Experimental Procedures

1. Subjects and behavioural method

Male albino Wistar rats, one year old, weighing 350-380 g were used as subjects.

The animals were housed in individual 'metabolism cages' and had food and water ad libitum (Ehret, Emmendingen, West Germany) 48 h before the first collection of urine the animals were transferred from their home cages to the metabolism cages.

The animals were divided into two groups: the 'manager' and the 'yoked control' group (n = 3 per group). To train the two groups, six identical plexiglas shuttle-boxes were used, 21 cm high, 17 cm deep and 47 cm wide. Scrambled shock-pulses (0.8 mA effective constant ac-current, 2 pulses per sec.) were delivered via a stainless steel grid (39 rods, 1.2 cm apart).

The boxes were enclosed in sound attenuating chambers. The presentation of shock stimuli and the registration of the animal responses were controlled by a programmable digital system (Demel, Hilden, West Germany) and recorded on a printer.

The manager and yoked control animals were exposed to the shock treatment for 30 days, with one 60 min session per day. Each trial consisted of a 20 s unsignalled shock exposure time and a variable shock-off interval having an average duration of 30 s. This variable shock-off interval schedule was used to avoid conditioning the animals. During the shock exposure time the manager animals could terminate the shock by escaping to the other half of the shuttle-box (FR-1 escape schedule). Their respective yoked controls were subjected to the same amount of shock determined by the manager animals, but could not avoid it. Thus a yoked animal was comparable with its respective manager animal in the amount of noxius stimuli it got, but not in the amount of controllability of those stimuli.

After each session - occuring simultaneously for each manager-yoked control pair (8-10 a.m.) - the immediately frozen urine samples were collected and stored at -18° C.

## 2. Materials

The trimethylsilylating reagent MSTFA* and the N-acylating agent MBHFBA* were supplied by Macherey & Nagel (Düren, West Germany). The deuterium labeled biogenic amines and metabolites were obtained from Merck, Sharp & Dohme (Point Clare, Canada) and Brucker & Franzen (Bremen, West-Germany).

The separation of the O-trimethylsilyl N-heptafluorobutyric derivates were achieved on 25 m long 0.3 mm I.D. and 0.4 mm layer thickness SE-30 capillary column from LKB (Bromma, Sweden) and Brucker & Franzen (Bremen, West Germany). The GC-injection was performed splitless with a modified moving needle system from Chrompack (Middelburg, Nederland). Detection was performed using a LKB 2091 Gaschromatographmass-spectrometer equipped with a jet-separator and a programmable 16 channel multiple ion monitor. Details were published separately (Kobelt, Wiesener, 1978).

## 3. Analytical Procedures

### 3.1 Sample collection

Urine specimens were collected each day as 23 h samples in cooled tubes (-10°C) before the 1 h foot-shock session. The frozen samples were allowed to melt, the urine volume measured and stored at -18°C.

### 3.2 Sample preparation

The simultaneous determination of unconjugated urinary biogenic amines and metabolites (A*, NA*, MNA*, HMPG*, DHPG*, p-Tyra*, m-Tyra*, GABA*) were performed using a modified version of the assay method previously developed in this laboratory (Kobelt, Wiesener, 1978, 1980). The compounds were submitted to a O-TMS* and N-HFB* derivatisation (Doenicke, 1975). Deuterium and nondeuterium standards were dissolved in 100 µl AN*, 10 µl MSTFA* (O-trimethylsilylation) and in 5 µl MBHFBA* (N-heptafluorobutyrycacylation).

The time required for dissolution was approximately 12 h at room temperature in tightly closed vials under anhydrous conditions and argon atmosphere.

The stock solution was stable for at least 6 months at 4°C. Fig. 1 shows a gaschromatogram of the derivatized biogenic and metabolites in the nanogram range.

To each 10 % aliquot of the 23 h urine specimens 10 µl of a deuterium standard solution, acting as internal standard was added. The µl amount contains $D_3$-A, $D_3$-NA = 100 ng, $D_3$-HPMG = 600 ng, $D_4$-p-Tyra = 2000 ng and $D_6$-GABA = 3000 ng. Afterwards the urine samples were lyophilisated, the dry residues were reacted with the derivatisation agent as already described. The subsequent GC-MF* assays were carried out with a 3 ul aliquot.

3.3 GC-MF* conditions

The GC-run was performed using the following temperature program: 155°C start, 5 min. isotherm and 5°C/min. slope. The injection part temperature was 180°C, separator 220°C and the ion source 230°C. The carrier gas (helium) flow rate was 2 ml/min., the 'make up' gas rate 30 ml/min., the electron energy 70 eV and the emission current 50 mA.

3.4 GC-MF* determination

Fig. 3 shows a gaschromatogram with mass-fragmentographic detection of a rat urine sample. The mass traces of the endogenous and deuterium analogue compounds were monitored in time gates according to their respectively retention time.

Fig. 1

Separation of biogenic amines and metabolites as O-trimethysilyl and N-heptafluorobutyrylacyl derivatives on a 25 m SE-30 capillary column.

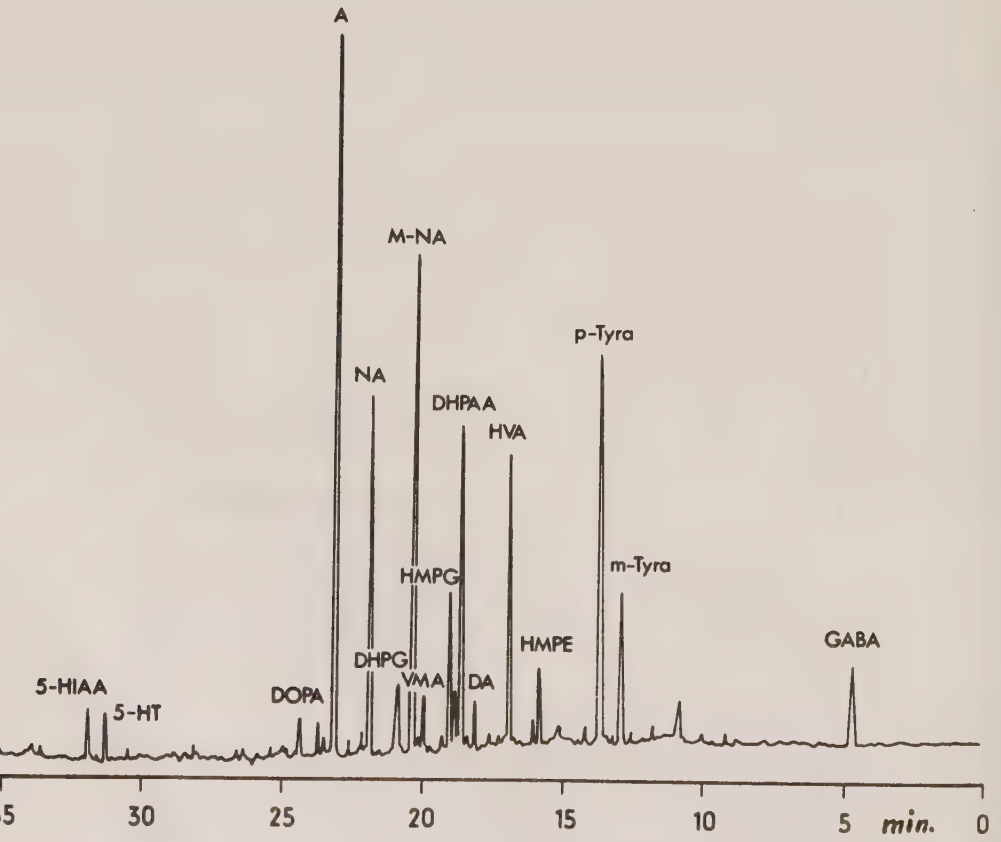


Fig. 2

Mass spectra of p-Tyramine and $D_4$-p-Tyramine as O-trimethylsilyl and N-heptafluorobutyrycacyl derivative.

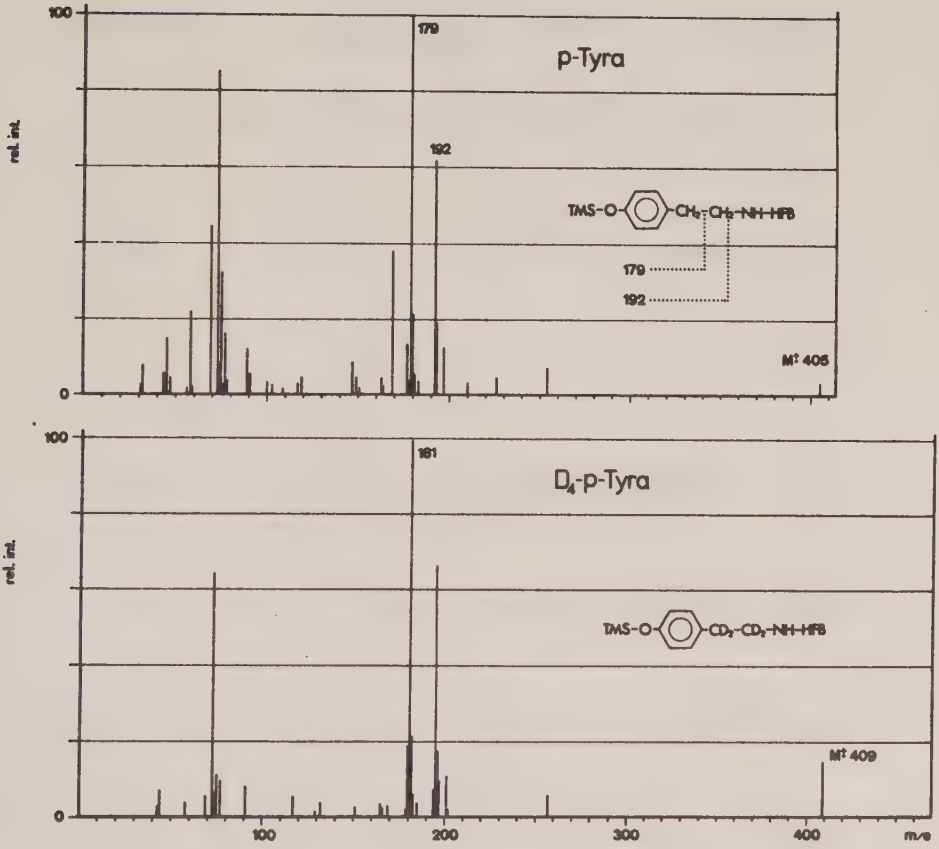


Table 1

Most abundant mass fragments of detected biogenic amines and metabolites and their deuterium analogues as O-trimethylsilyl N-heptafluorobutyrycacyl derivatives.

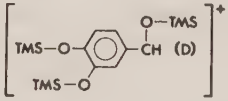
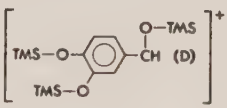
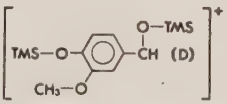
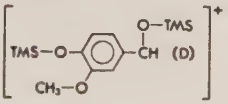
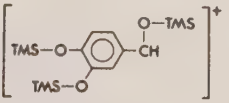
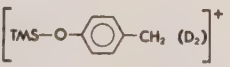
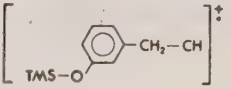
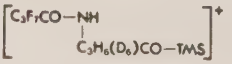
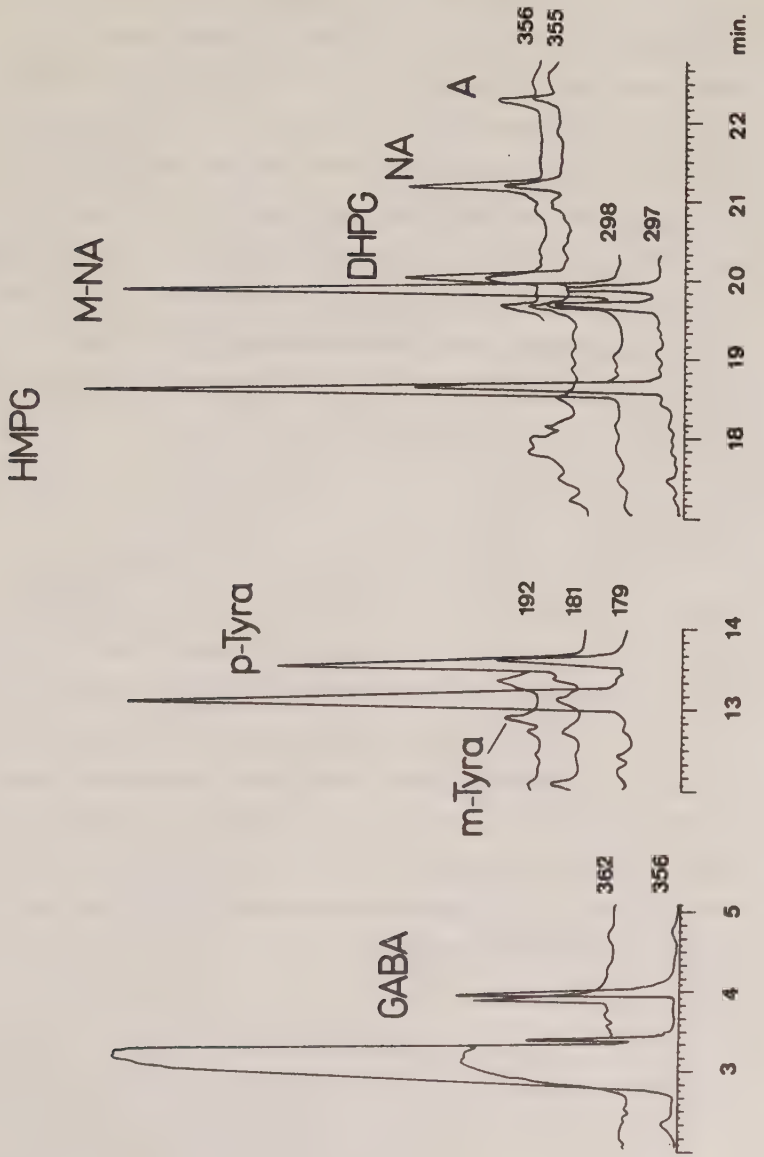
| Compound | m/e | Detected Ion Structure | | Ret.Time |
|---|---|---|---|---|
| A / $D_3$-A | 355/356 |  | $M^{+}-240$ | 22' |
| NA / $D_3$-NA | 355/356 |  | $M^{+}-226$ | 21' |
| M-NA / $D_3$-M-NA | 297/298 |  | $M^{+}-226$ | 20' |
| HMPG / $D_3$-HMPG | 297/298 |  | $M^{+}-103$ | 18' 30" |
| DHPG / $D_3$-HMPG | 355/298 |  | $M^{+}-103$ | 20' 10" |
| p-Tyra / $D_4$-p-Tyra | 179/181 |  | $M^{+}-226$ | 14' |
| m-Tyra / $D_4$-p-Tyra | 192/181 |  | $M^{+}-213$ | 13' |
| GABA / $D_6$-GABA | 356/362 |  | $M^{+}-15$ | 4' |

Fig. 3

Mass fragmentogram demonstrating simultaneous determination of biogenic amines and metabolites as O-trimethylsilyl N-heptafluorobutyrycacryl derivates in urine, with their corresponding deutero analogue compounds, detected by multiple ion monitoring in time gates.



The quantification followed by the peak height ratio method, using the most abundant positive ion (table 1, fig. 2). The linearitiy of responses was determined using calibration curves (nondeuterium/deuterium ratio versus different amounts on nondeuterium standards). Recovery rates were not determined as the chemical similarity of the assayed endogenous compounds and their deuterium analogues were used as internal standards.

The accuracy of the assay was determined for 15 measured points (30 days measured each second day) with four different aliquot specimens of the pooled 23 h urine samples. The coefficient of variation from these repeated measurements (n = 4 over 15 measure points) shows values between 4 - 11 % for the different compounds.

4. Statistical analysis

For the computer-aided statistical analysis of the urine excretion profiles a 3-factor analysis of variance with repeated measurement on two factors was used (Winer, 1971, 539 pp), which ist part of the program package BMDP 2V. There were two independent groups of animals (manager vs. yoked control animals), whose daily urine output was sampled, defined the treatment factor. 15 time points determined every 2nd day and representing the mean of 4 aliquot samples (as specified in '3.4 GC-MF-Determination'), defined the first factor of repeated measurement.

The second repeated measurement factor was constituted by the biochemical variables.

To consider them as realizations of a repeated measurement factor was possible for two reasons: 1. They were true correlated variables - repetitively determined from the same urine probe. 2. They were quantitatively comparable with one another based on their internal deuterium standardization. To despict possible trends effects for the various biochemical profiles or the mean profile of the combined biochemical variables, trend-tests were performed for the first ten orthogonal components. The daily urinary volume output was evaluated with a separate 2-factor analysis of variance with the treatment-variable as an independent factor and the 30 days of experimentation as realization of a repeated measurement factor.

## 5. Results

### 5.1 Behavioural responses

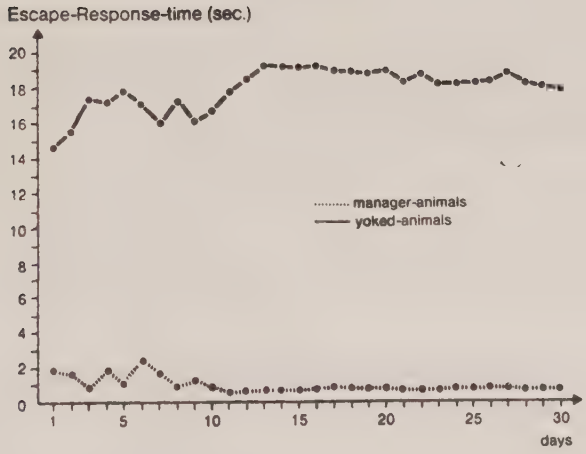
Fig. 4 shows the escape response for the two experimental groups.

From the very beginning the manager animals show a high performance rate indicating an almost immediate escape after the first shock-pulse (shock-pulse frequency: 0,5 / s). This rapid acquisition of the escape-response is quite typical for rats in a shuttle-box situation as it usually takes no more than 10 - 15 trials to show a reliable escape-response when the shock is presented. The yoked animals - after a slight increase in escape response time - show practically no response tendency.

Fig. 4

Latency time between the onset of the shock trials and the successful performance of the escape-response for the yoked(n = 3)and manager(n = 3) animals.

Each point represents the group mean of fifty trials.



5.2 Biochemical results

For the pooled catecholamines and metabolites (fig. 5) the analysis of variance and the trend analysis showed various significant time effects. This analysis revealed that the main effect of the time variable ($p \leq 0.001$) was mainly attributable to the influence of a significant linear ($p \leq 0.01$) quadratic ($p \leq 0.01$) and cubic ($p \leq 0.01$) trend component. The curve fit was made by the least square method using only significant trend components ($p \leq 0.05$) for the polynomial model.

Both groups, yoked and manager animals, increase their catecholamine output at the end of the test period, indicating another maximum beyond the $30^{th}$ day.

The excretion pattern of the catecholamines and metabolites as pooled and individual biochemical variables do not show any statistically significant differances between the two animal groups.

Fig. 6 shows the single catecholamines and metabolites urinary output.

The excretion patterns of the yoked and manager groups Adrenaline and Normetanephrine suggest a marked monophasic trend with a maximum of activity between the 4th and the 12th day. In contrast, Noradrenaline shows evidence of a biphasic trend pattern at a first maximum as does Adrenaline and Normetanephrine, and at the second maximum between the 20th and 24th day. It should, however, be noted that HMPG and DHPG show a peak of activity at this second maximum.

p-Tyramine and GABA (fig. 7) confirm the general observation that a specific maximal activity is displayed around the 10th and between the 20th and 24th day. p-Tyramine is qualitatively different from the other biogenic amines, as it has a significant linear increase based on time ($p \leq 0.01$).

Fig.8 shows the absolute amount of urine excretion for the two groups as a function of time. the obvious increase in the total amount of urine output over a period of time is statistically confirmed by a significant effect of the time factor ($p \leq 0.01$) in the analysis of variance.

p-Tyramine and the water diuresis indicate differences between the yoked and manager animal groups ($p \leq 0.1$) at the last experimental days, which probably could be confirmed statistically.

## 5.3 Histological findings

The histological findings after the animals decapitation showed for the adreno medullary cells an extensive fatty infiltration and large vacuolisation without differences between the yoked and manager animal groups.

Fig.5

Urine excretion profile of the pooled catecholamines, unconjugated metabolites for the yoked (n = 3) and manager (n = 3) animals.
For the scaling of the axis and the curve estimation procedure see fig. 5.

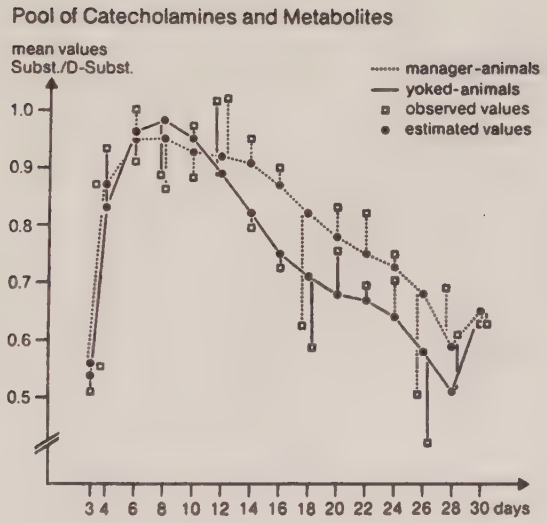


Fig.6

Urine excretion profile of the single compounds, catecholamines and unconjugated metabolites. Each urine speciment represents the mean of repeated measurements (n = 4), each measurement point the mean ± SD of the yoked □ (n = 3) and manager △ (n = 3) animals. For technical reasons the stress application started with the 3rd experimental day.

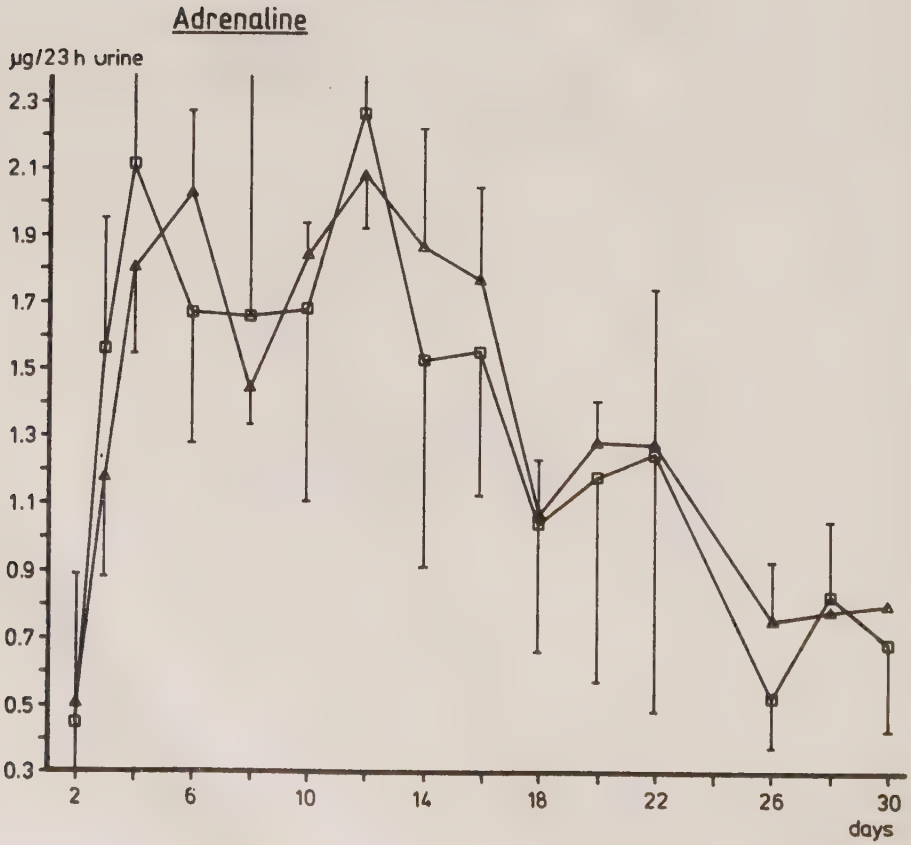
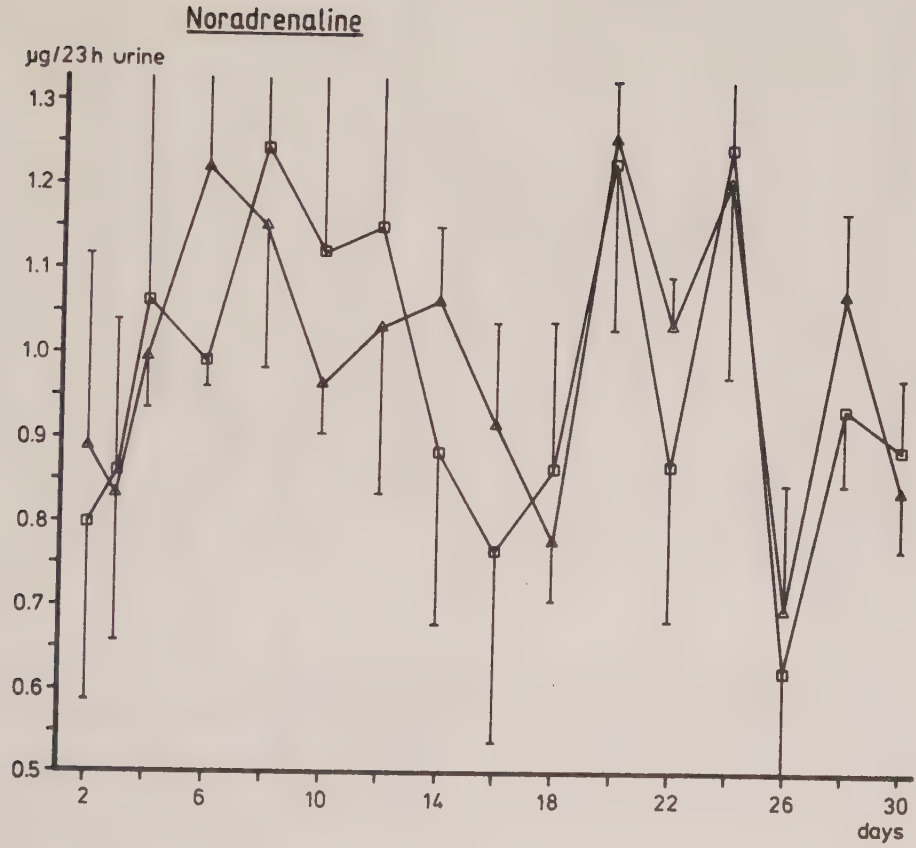


Fig.6 Continuation



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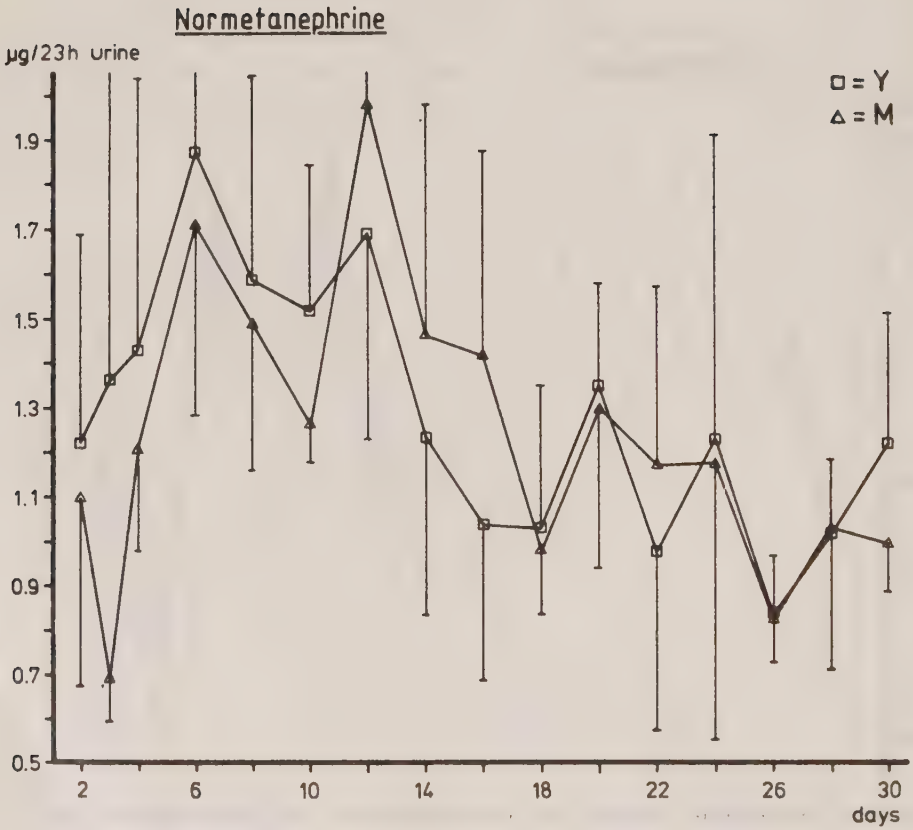

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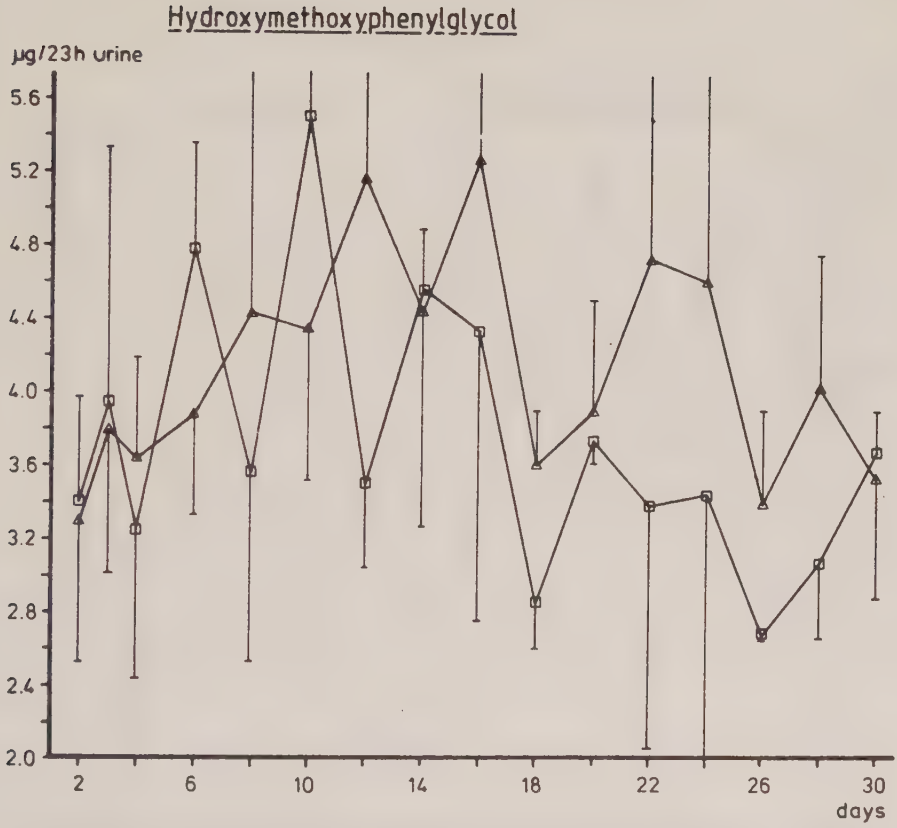


Fig.5 Continuation

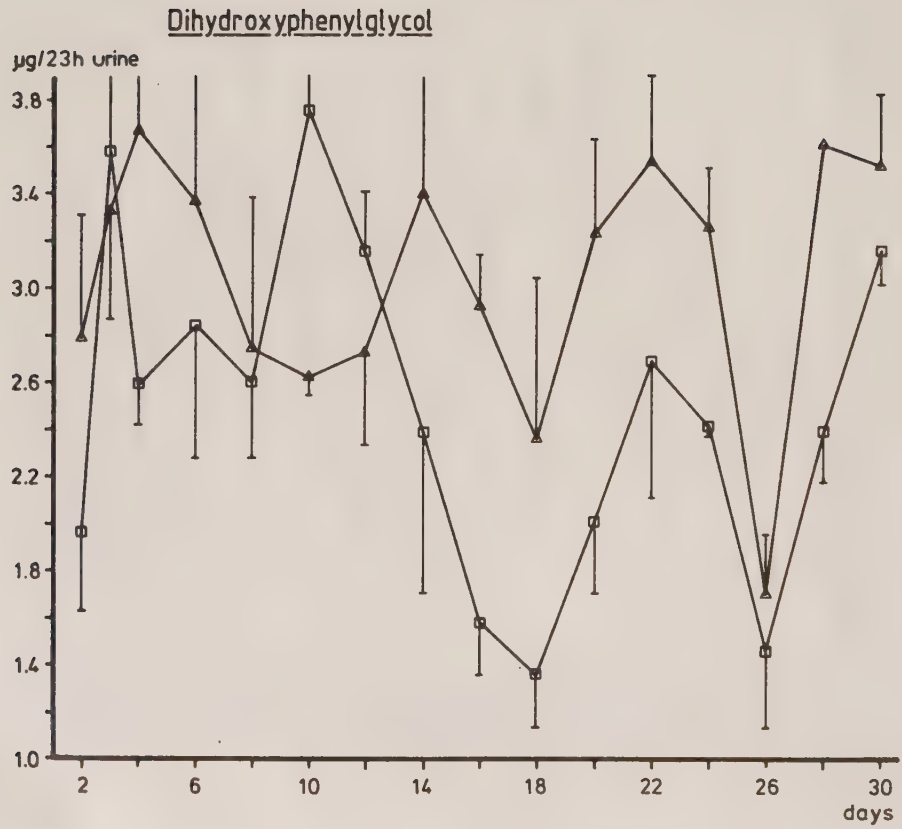


Fig.7

Urine excretion profile of the assayed non catecholamine compounds. Each urine speciment represents the mean of repeated measurements (n = 4), each measurement point the mean ± SD of the yoked □ (n = 3) and manager △ (n = 3) animals.

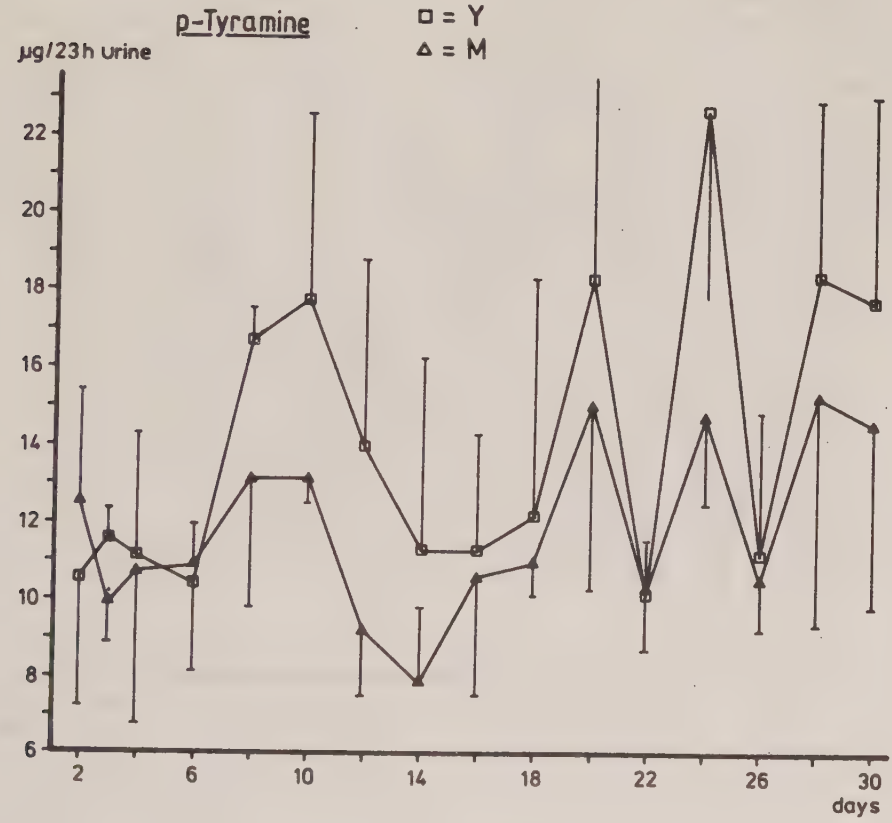


Fig.7 Continuation

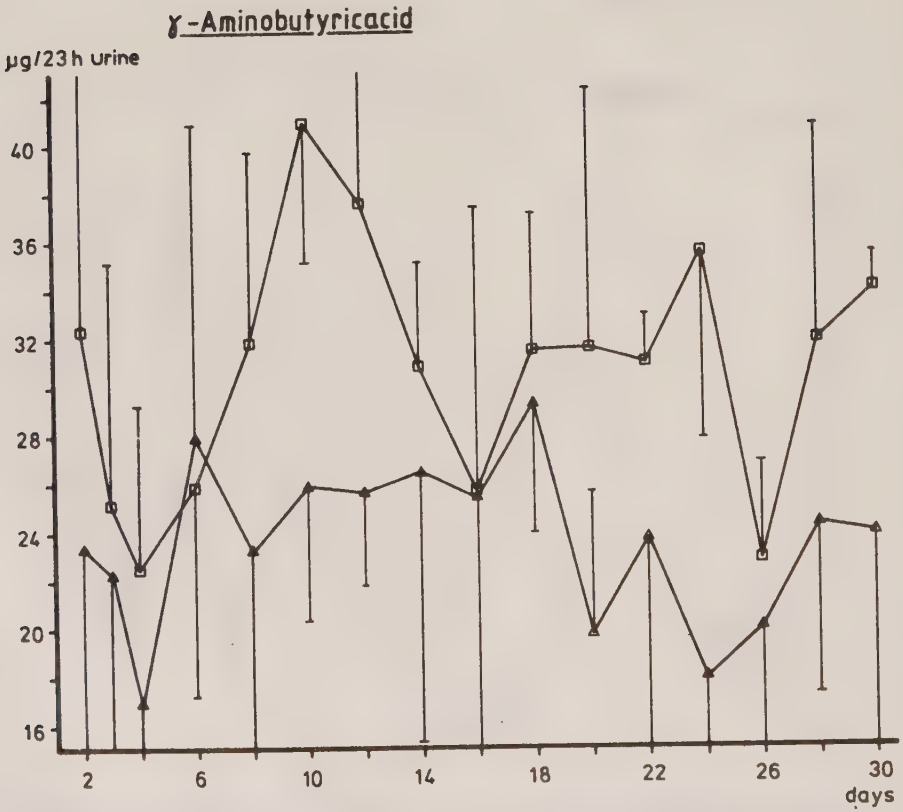
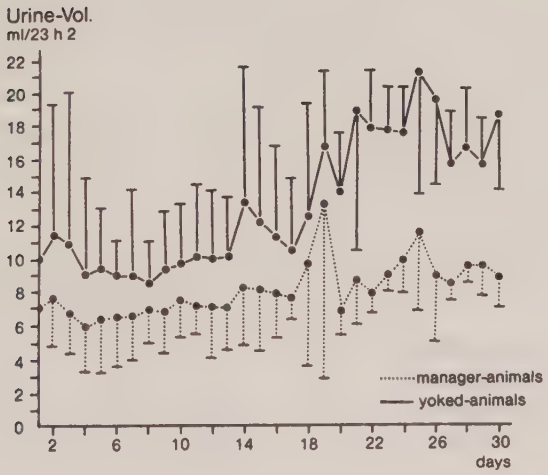


Fig. 8

Daily urine volumen output for the yoked (n=3) and manager (n=3) animals. Values are means ± SD.



## 6. Discussion

The first question we tried to answer in the present study was aimed to reveal a metabolical effect of the controllability factor.

The answer now cannot be given very clearly, as the only relevant result - a statistical tendency in the time x treatment interaction - showed up only for the pooled variables but could not affirmated in the more specific behaviour of the single biochemical variables. An effect of the treatment variable can therefore - at this stage of our research - only hypothetically assumed, rendering a more throughgoing exploration of this dimension to further experiments.

In regard to the remaining experimental questions, asking for common and compound related time effects, more specific answers can be given.

For the pooled catecholamines and metabolites following a three-phase-model of time development can be applied: after the onset of the shuttle-box session a rise of neurohumeral activity leads to a first maximum (phase I) up to the 8th day. The course of activitiy decreases and goes through an intermittent state of change (phase II) from th 8th day to the 28th day. After a state of minimal activity - comparable to the activity level at the onset - a third change takes place (phase III). The extension of the experiment beyond the 30th day would probably have revealed a further excretion maximum and presumbly would have led to the death of at least some of the animals.

Taken into account the adrenal medullary histological findings, it is reasonable to assume that the animals had already reached a state of exhaustion (phase III).

The general biochemical activity principally follows the same time model and gives an insight into the trend pattern common to all variables.
Beside a linear and quadratic trend component (both negative) the former related to the generally decreasing tendency, the latter related to the existence of the strong maximum on day 8 - 10, a cubic trend component indicates the tendency to show an increase after the foregoing maximum. This cubic behaviour could sionificantly revealed by trend analysis in 5 of the 7 variables ( 5 catecholamines and metabolites).

Selye (1975) describes the general adaption syndrome to a continuous stressor in the sense of a cubic time model: after an increase of stress-related neurochemical activity (alarm reaction) a more or less prolonged phase of reduced response is observed (resistant reaction). Before a functional breakdown takes place, the organism shows an increase in neurochemical activitiy to mobilize energy resources in a final stage of exhaustion.

Our results generally agree with the cubic model as described by Selye (1975). We further provide evidence that there may be intermittent phases of activity (phase II), which indicate that the regulation of neurochemical adaption to a chronic stress situation may follow a polyphasic time pattern.
An analysis of the excretion pattern of the single variables may help to understand to what extend the different compounds contribute to the polyphasic behaviour of the pooled activity.

The adrenaline output resembles a more monophasic trend pattern in the stage II (resistent stage) whereas noradrenaline has in this stage a biphasic pattern which is replicated to a certain extend in the time behaviour of the conjugated glycolic metabolites (HMPG, DHPG).

Similar differential time effects for adrenaline and noradrenaline during chronic exposure to stress situations were published earlier (Leduc, 1961; Le Blanc, 1976; Benés, 1978).
The behaviour of adrenaline seems to follow more closely the time model developed by Selye than noradrenaline does. The explanation may be that with noradrenaline different systems are involved in the synthesis and regulation of the compound.
It is a well established fact that adrenaline is mainly produced by the chromaffin cells of the adrenal medulla.
In the rat, noradrenaline is produced to about 40 % by the adrenal glands, and about 60 % by the nerve ending of the sympathetic system (Mc Carty et al., 1979). According to the published NA-excretion data during chronic stress exposure (Zigmond et al., 1970; Thierry et al., 1968; Weiss et al., 1975) the second maximum during stage II seems to be due to the influence of the sympathetic nervous system. Even the excretion pattern of normetanephrine suggests a similar conclusion.
Normethanephrine (MNA) is one of the principal metabolites of noradrenaline (NA) and is found in large concentrations in the adrenomedullary tissue of the rat (Anton, 1966).

The comparison of the excretion pattern of MNA and NA shows that the second maximum of MNA during the stage II phase is not very pronounced.

It is therefore not implausible to assume that the normethanephrine-excretion reflects the noradrenaline-adrenomedullary fraction which has a similar excretion pattern as adrenaline.

Furthermore, this would agree with the lack of normetanephrine enhancement in the spinal cord after restraint stress (Takagi et al., 1979). There is a considerable difference between the catecholamine output and p-Tyramine and the urine volume. Both p-Tyramine and the urine volume show a slow oscillatory enhancement over the observed time. Furthermore p-Tyramine and GABA show a decline after the first stress session. The diuresis by chronical exposure to stress events is in partial agreement with earlier results (Leduc, 1961; Schmucki, 1979) who described a longlasting enhancement after an initial urine decrease.

As already mentioned it has not been possible to statistically establish different excretion levels for the single substances comparing the yoked and manager animals. This was probably due to the limited number of animals used. This handicap was implicated by the preliminary screening work using a sophisticated analytical tool which provided us with data on a large number of different parameters.

Possible differences between yoked and manager animals suggest that the discrimination would be more pronounced in a larger stress stimulated biological system. The possibly higher diuresis increased by the lack of stress control may be due to differences in the corticosterone secretion (Coover et al., 1973) or by possible variations in the amount of vasopressin output and hypothalamic noradrenaline depletion (Brownstein et al., 1979; Knepel et al., 1982).

Interpretations of the p-Tyramine results must at this time be very speculative, taking into account that the nature, the origin and the function of p-Tyramine are still being controversially discussed (Boulton, 1976; Huebert et al., 1978; Fellman, 1978). Apart from the catecholamine levels further specific stress work has to include the p-Tyramine output.

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